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Short communication

Effect of fat in ground beef on the growth and virulence plasmid (pYV) stability in *Yersinia pestis*☆

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ABSTRACT

Knowledge of the behavior of *Yersinia pestis* in food may be useful in the event *Y. pestis* is used in a bioterrorism attack on the food supply. However, there are no reports on the growth of plasmid-bearing (pYV) virulent *Y. pestis* in food. The growth of a conditionally virulent pYV-bearing *Y. pestis* KIM5 in sterile raw ground beef with 7, 15 and 25% fat content was studied at 0, 4, 10 and 25 °C. The *Y. pestis* KIM5 did not grow but survived in raw ground beef at 0 and 4 °C. In raw ground beef with fat contents of 7, 15 and 25% *Y. pestis* KIM5 replicated at 10 °C with growth rates of 0.06, 0.05, and 0.06 $\log_{10}$ CFU/h and maximum population densities of 8.65, 8.30, and 8.43 $\log_{10}$ CFU/g, respectively. The growth rate was 4-fold higher and the maximum population density was slightly higher at 25 °C in raw ground beef at all levels of fat as compared to 10 °C. Moreover, there was no loss of pYV in surviving *Y. pestis* KIM5 in raw ground beef stored at refrigerator temperatures or during its growth in raw ground beef. This suggests that raw ground beef contaminated with virulent *Y. pestis* could cause oro-pharyngeal plague due to refrigeration failure, temperature (10–25 °C) abuse, and if the meat was not properly cooked. The resultant disease may lead to outbreaks of highly infectious pneumonic plague.

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1. Introduction

The genus *Yersinia* consists of 11 species, but only *Yersinia pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* are considered to be pathogenic to humans. Among these three human pathogens, *Y. pestis*, the causative agent of bubonic plague in humans is considered the most invasive and virulent and has been described as one of the most devastating infectious agents in world history. *Y. pestis* has also been proposed to be ancestrally related to *Y. pseudotuberculosis*, but the latter exhibits similar behaviors and clinical symptoms to the more distantly related *Y. enterocolitica* (Wren, 2003). While rare, *Y. pestis* has been implicated as a foodborne pathogen causing foodborne outbreaks of oro-pharyngeal plague by the handling or consumption of inadequately cooked goat and camel meat (Arbaji et al., 2005; Bin Saeed et al., 2005; Christie et al., 1980). The risk, morbidity, and mortality of contracting plague through the consumption of food deliberately contaminated with *Y. pestis* are currently unknown but potentially real. Furthermore, the identification of multidrug-resistant strains (Gailmand et al., 1997) and the potential use of this pathogen in food for the deliberate contamination of food could cause plague in large populations.

Three virulence plasmids [pYV (70.3-kb, Yops, type III secretion system), pFra/pMT1 (96.2-kb, murine toxin: phospholipase, F1 capsule-like antigen), and pCP1/pPst/pPla (9.6-kb, plasminogen activator)] are involved in the virulence of *Y. pestis* (Brubaker, 2006; Bearden and Perry, 2008). Among these three plasmids, the type III secretion system (Yops) encoded by pYV promotes cytotoxicity and the common symptoms of plague (Brubaker, 2006). Furthermore, the pYV in *Y. pestis* has been correlated with several other phenotypes including calcium-dependent growth (low calcium response: Lcr, pin point colony of the size 0.36 mm), crystal violet (CV) binding (dark-violet colony), and Congo red (CR)-uptake (red pin point colony, size = 0.36 mm) which are expressed at 37 °C but not at 28 °C (Bhaduri and Sommers, 2008). These pYV encoded phenotypes were used to identify this virulence plasmid in pathogenic *Yersinia* species (Bhaduri, 2001; Bhaduri and Sommers, 2008). However, the pYV is known to be unstable (Bhaduri, 2001; Brubaker, 2006; Carniel, 2006; Robins-Browne, 2001; Skurnik et al., 2002). In general, cells lose pYV with subculture and during storage at refrigerator/freezing temperatures as well as at incubation temperatures over 30 °C. Loss of this plasmid results in loss of virulence and the concomitant disappearance of the associated phenotypic characteristics.

A previous study on the growth of *Y. pestis* in raw ground beef was done using a derivative of *Y. pestis* KIM5 lacking the pYV virulence plasmid (Tamplin and Bhaduri, unpublished data). The growth rate of

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this pYVless avirulent *Y. pestis* KIM5 is much higher than pYV-bearing conditionally virulent *Y. pestis* KIM5 (Bearden and Perry, 2008; Bhaduri and Sommers, 2008). Ground beef is produced in large quantities and, if contaminated, could affect the health of numerous consumers. The resulting ground product is then distributed throughout wholesale and retail markets in chubs and hamburger patties. Exposure assessments for foodborne disease require information about the behavior of pathogens for various processing and handling conditions that occur throughout the food chain. In this regard, there are few available reports concerning the fate of *Y. pestis* in foods, and more specifically, in ground beef. Previously, it was demonstrated that increased fat content inhibits the growth of *Y. enterocolitica* in meat samples (Bhaduri et al., 1997; Kwaga and Iversen, 1991). Hence, it is important to know how different levels of fat in raw ground beef affect the growth of *Y. pestis* KIM5. In this study, experiments were conducted to monitor the growth of pYV-bearing conditionally virulent *Y. pestis* KIM5 in raw ground beef with 7, 15, and 25% fat over a range of temperatures. Moreover, to fully assess the potential risk of illness, it is necessary to know the stability of the pYV in *Y. pestis* during its growth in raw ground beef.

2. Materials and methods

2.1. Bacterial strain

Y. pestis KIM5, a derivative of strain KIM (Kurdistan Iran man) which lacks the chromosomally-encoded pigmentation (Pgm^-) locus, but contains all three virulence plasmids (Bearden and Perry, 2008), was used in this study. This strain is conditionally virulent (a conditional mutant is only infectious if inoculated intravenously) and can be used in a BL2 laboratory facility (Bearden and Perry, 2008). This is the only *Y. pestis* strain with all three virulence plasmids available and was kindly provided by Dr. Susan Straley (Department of Microbiology and Immunology, University of Kentucky, Lexington, KY). This strain is well characterized, and has been extensively used to study the microbiology and molecular pathogenesis of this bacterium (Bearden and Perry, 2008; Bhaduri and Sommers, 2008; Brubaker, 2006). Storage of cultures, preparation of inocula, and incubation conditions have been described previously (Bhaduri and Sommers, 2008). The presence of pYV was confirmed by low calcium response (Lcr), Congo red (CR) binding, and PCR assay targeting a key regulatory gene, *virF*, present on pYV (Bhaduri, 2003; Bhaduri and Sommers, 2008).

2.2. Sample inoculation

Packages of raw ground beef containing approximately 7, 15 and 25% fat were purchased at a local supermarket and were irradiated as described previously (Bhaduri and Phillips, in press]). Triplicate 3-g (+/−0.2 g) portions of sterile irradiated raw ground beef from three different separate batches were thawed and were placed in individual 100-ml capacity filter stomacher bags (A. J. Seward, London, UK). For each batch, 350 µl of a 10^4 CFU/ml suspension of *Y. pestis* KIM5 in 1% peptone water were added to thawed raw ground beef. Thus, the final concentration of *Y. pestis* KIM5 in raw ground beef was ~10^3 CFU/g. The inoculum was hand-massaged into the ground beef through the stomacher bag for ~30 s, followed by stomaching (Model Bag Mixer 400, Interscience Inc., Weymouth, MA) the sample for 2 min at room temperature. The bags were loosely sealed with tape to permit ambient air exchange.

2.3. Growth study

There are no data concerning the growth of *Y. pestis* in raw ground beef with 7, 15, and 25% fat during storage over a range of temperatures. In this study, *Y. pestis* KIM5 was initially grown in BHI broth at 25 °C for 18 h and diluted to 10^4 CFU/ml in 1% PW and then *Y. pestis* KIM5 was inoculated into radiation sterilized raw ground beef to study its growth at 0, 4, 10 and 25 °C (temperatures which are found in commercial and consumer handling practices). Storage at 0 and 4 °C will determine whether *Y. pestis* KIM5 is cold-tolerant. Refrigerator temperature has been shown to vary from top to bottom with maximum temperature of 17.2 °C (www.foodrisk.org: EcoSure, 2008); hence, the growth of *Y. pestis* KIM5 was also monitored at10 °C. The temperatures above 28 °C facilitate the loss of pYV which results in avirulent cells (Bhaduri and Sommers, 2008). Thus, the growth of *Y. pestis* KIM5 strain was not studied above 25 °C. Thus the growth of *Y. pestis* KIM5 was studied at 0, 4, 10 and 25 °C in sterile raw ground beef. The samples were grown at the respective temperature until they attained a stationary phase with 11–15 data points. High precision (±0.01 °C) temperature-controlled circulating water baths (model RTE 17, Thermo Neslab, Newington, NH) were used to maintain the temperatures at 0, 4, 10 and 25 °C. Two trials from separate batches of sterile raw ground beef were conducted for each growth temperature, with triplicate samples per time point and triplicate plating per sample after serial dilution. The water temperatures were continuously monitored using a calibrated temperature data logger (model FT121 or D100, Dickson, Addison, IL) as described by Bhaduri and Phillips (in press).

2.4. Enumeration

At each time interval, three samples of inoculated raw ground beef were diluted with 1% peptone water in 10-fold serial increments, stomached for 2 min, 0.05 ml was surface-plated on Congo red (CR) magnesium oxalate agar (CRMOX), and the plates were incubated at 37 °C for 24 h. Red pin point colonies (expression of Lcr and CR-binding phenotypes) appearing on agar plates indicated the presence of the pYV-bearing *Y. pestis* KIM5 (Bhaduri and Sommers, 2008). Colonies were counted using a ProtoCOL colony counter with version 3.15.630 software (Protocol PC Model 66000, Microbiology International, Frederick, MD). Data were transferred to an Excel® spreadsheet for analysis (Microsoft Corp., Redmond, WA).

2.5. Data analyses

DMFit, using the Baranyi dynamic growth model, was used to fit the data time-versus-$\log_{10}$ CFU plots which were used to measure growth rate and maximum population density (Baranyi et al., 1993).

3. Results and discussion

3.1. Growth rate

The *Y. pestis* KIM5 strain did not grow in raw ground beef with 7, 15 and 25% fat at 0 and 4 °C storage during a 2 month period but did survive under these conditions (Fig. 1). Therefore, the survival of *Y. pestis* KIM5 at 0 and 4 °C in all three types of raw ground beef suggests a potential health risk for refrigerated foods containing *Y. pestis*. The *Y. pestis* KIM5 strain grew in raw ground beef at storage temperatures of 10 and 25 °C. No lag phase was observed within the sampling time intervals. At 10 °C in all fat levels the differences in growth rates were insignificant. The growth rate ($p<0.001$) increased from 0.057, 0.051, 0.055 $\log_{10}$ CFU/h at 10 °C to a rate of 0.233, 0.205, 0.196 $\log_{10}$ CFU/h at 25 °C in raw ground beef containing 7, 15 and 25% fat, respectively (Fig. 1). Thus, the growth rate increased 4-fold in all three fat levels when the storage temperature increased from 10 to 25 °C (Fig. 1). However, the fat content of raw ground beef had no effect ($p>0.05$) on the growth of *Y. pestis* KIM5 in contrast to what was reported previously in pYV-bearing *Y. enterocolitica* (Bhaduri et al., 1997; Kwaga and Iversen, 1991).

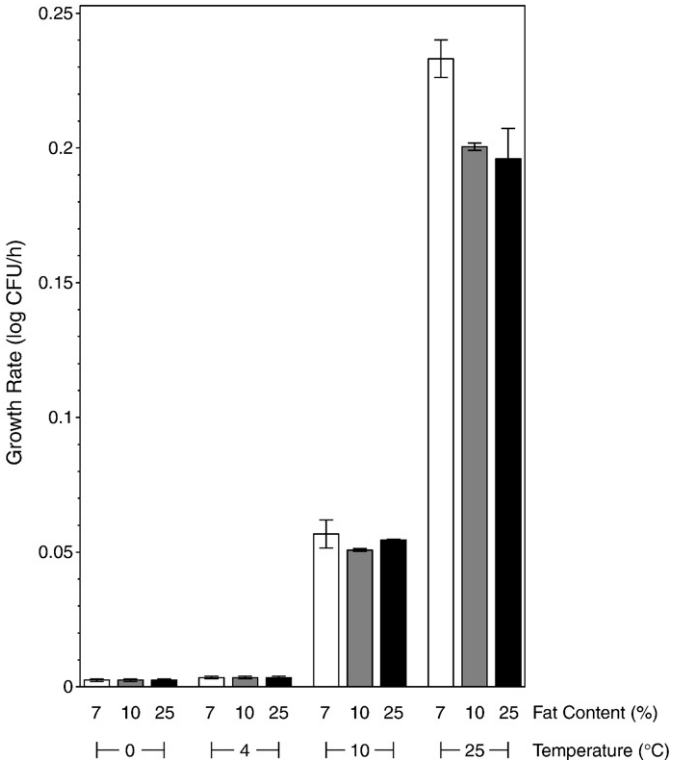

Fig. 1. Effect of fat content on the growth rates of pYV-bearing *Y. pestis* KIM5 in sterile raw + ground beef as a function of storage temperature at 0, 4, 10 and 25 °C. Presence of pYV was indicated by the appearance of red pin point colonies on CRMOX. Error bars (I) indicate values within 1 standard deviation of the mean.

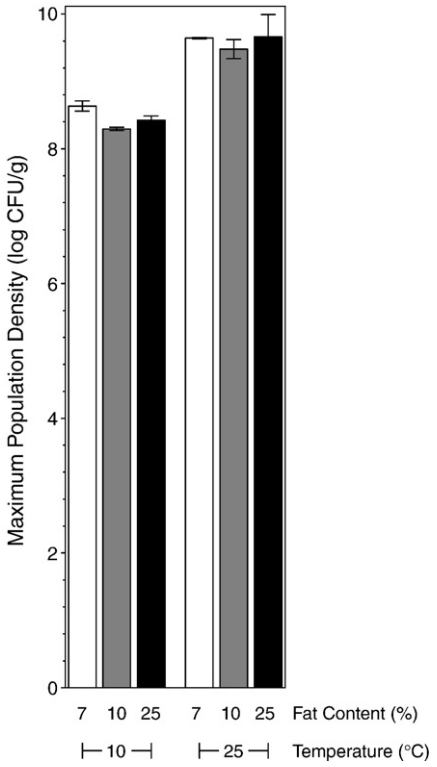

Fig. 2. Effect of fat content on the maximum population density of pYV-bearing *Y. pestis* KIM5 in sterile raw ground beef as a function of storage temperature at 0, 4, 10 and 25 °C. Error bars (I) indicate values within 1 standard deviation of the mean.

The growth rate of *Y. pestis* KIM5 is in general agreement with its growth in BHI broth at 10 and 25 °C (data not shown). There is no literature available to compare the growth rate of *Y. pestis* KIM5 in raw ground beef with its growth in other foods. Since *Y. pestis* and *Y. pseudotuberculosis* are closely related (Hinchliffe et al., 2003; Achtman et al., 1999; Wren, 2003), the growth rate of *Y. pestis* KIM5 was compared with the previously reported (Bhaduri and Phillips, in press) growth rate of pYV-bearing *Y. pseudotuberculosis* at 0, 4, 10, and 25 °C in raw ground beef with 7% fat. In contrast to *Y. pestis* KIM5, *Y. pseudotuberculosis* grew in raw ground beef at 0 and 4 °C (Bhaduri and Phillips, in press). The growth rate for these two pathogens was similar at 10 °C. However, the gowth rate of pYV-bearing *Y. pseudotuberculosis* was much higher ($p<0.01$: 0.620 $\log_{10}$ CFU/h) than *Y. pestis* KIM5 (0.233) at 25 °C.

3.2. Maximum population density (MPD)

The maximum population density represents the highest concentration that a microbial population attains in an environment. This level can be influenced by limiting quantities of nutrients and/or by production of inhibitory substances. Since *Y. pestis* KIM5 did not grow in raw ground beef containing 7, 15 and 25% fat at 0 and 4 °C, the maximum population density was not determined. The maximum population density of *Y. pestis* KIM5 was 8.7, 8.3, and 8.4 $\log_{10}$ CFU/g at 10 °C and 9.6, 9.5, and 9.7 $\log_{10}$ CFU/g at 25 °C in raw ground beef containing 7, 15 and 25% fat, respectively (Fig. 2). There were no significant differences ($p>0.05$) in the maximum population density at 7, 15 and 25% fat levels in raw ground beef at both 10 and 25 °C. Thus, the *Y. pestis* KIM5 maximum population density was a function of temperature, but not of raw ground beef fat content. The maximum population density of both *Y. pestis* and *Y. pseudotuberculosis* was similar at 10 °C, whereas, at 25 °C, the maximum population density of *Y. pseudotuberculosis* was somewhat higher ($p<0.08$: 1.5 $\log_{10}$ CFU/g difference) in raw ground beef with 7% fat (Bhaduri and Phillips, in press).

3.3. Stability of Y. pestis KIM5 during its growth in raw ground beef

Defining *Y. pestis* growth in food is not the only relevant information for risk assessment. In addition, it is important to understand the stability of genetic elements encoded by the chromosome and plasmids that are necessary for virulence of *Y. pestis* (Brubaker, 2006). The pYV in pathogenic *Yersinia* spp. is known to be unstable (Robins-Browne, 2001; Carniel, 2006; Skurnik et al., 2002). In general, cells lose pYV with subculture, and during storage at refrigerator or room temperatures. By using pYV encoded Lcr–CR-uptake phenotypic assay (appearance of red pin point colony) on CRMOX plates, it was found that pYV in *Y. pestis* KIM5 was stable in cells that survived during storage at 0 and 4 °C as well as those cells that grow at 10 and 25 °C in raw ground beef with fat levels ranging from 7 to 25%. This is the first study on the growth using a pYV-bearing strain of *Y. pestis* in food and this report showed that temperature abuse of *Y. pestis* KIM5 in food could cause a potential risk for the consumer.

In conclusion, *Y. pestis* KIM5 survives at 0 and 4 °C and grows in raw ground beef at 10 and 25 °C. The retention of pYV in *Y. pestis* KIM5 that survives at refrigerated temperatures could pose an increased health risk if retail raw ground beef is intentionally contaminated. It is also of great significance that *Y. pestis* KIM5 retained pYV during its growth in raw ground beef at common storage and handling temperatures. Therefore, raw ground beef contaminated with *Y. pestis* is potentially capable of causing oro-pharyngeal plague if it is stored under conditions such as refrigeration failure, temperature abuse (10 and 25 °C), and if the meat is not properly cooked. In addition, the individual infected by foodborne *Y. pestis* is a potential reservoir of *Y. pestis* who can infect others leading to outbreaks of highly infectious pneumonic plague.

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